Japanese Journal of Veterinary Research 67(2): 163–172, 2019

JJVR

REGULAR PAPER Experimental Research

Epidemiological survey of tick-borne encephalitis virus infection in wild animals on Hokkaido and Honshu islands, Japan

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Received for publication, October 3, 2018; accepted, November 14, 2018

Abstract

The first human case of tick-borne encephalitis (TBE) in Japan was recorded in southern Hokkaido in 1993 and was followed by four further cases in southern, central, and northern Hokkaido during 2016–2018. However, the distribution of TBE virus (TBEV) foci in Japan is unclear. Therefore, here, we serologically examined raccoons (*Procyon lotor*), sika deer (*Cervus nippon*), and wild boars (*Sus scrofa*) as sentinels of TBEV infection in Hokkaido and in Fukushima and Tochigi Prefectures in Honshu. A total of 1,649 serum samples collected between 2003 and 2018 were screened by enzyme-linked immunosorbent assay using subviral particles and confirmed using the virus neutralization test. In raccoons, the seroprevalence of TBEV was 5.9% (39/662 samples) in central Hokkaido in 2003–2005 and 0.8% (3/368 samples) in eastern Hokkaido in 2010–2018, revealing the presence of TBEV foci in these areas. In addition, 0.5% (2/414) of deer sampled in eastern Hokkaido in 2010–2017 and 2.4% (1/42) of deer sampled in Tochigi Prefecture in 2016–2018 were seropositive. On Honshu, seropositive rodents have previously been detected only in Shimane Prefecture. Therefore, the detection of seropositive animals in Tochigi Prefecture may indicate the widespread distribution of TBEV foci throughout Japan. TBEV and viral genes were not detected in 507 ticks collected in the same area of eastern Hokkaido where seropositive animals were found, reemphasizing the value of using serological examination of wild animals as a tool for revealing unknown TBE risk areas. Our findings also indicate that raccoons may be particularly useful sentinels.

Key Words: Deer, Raccoon, Sentinel, Seroprevalence, Tick-borne encephalitis (TBE)

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doi: 10.14943/jjvr.67.2.163

Introduction

Tick-borne encephalitis (TBE) is a tick-transmitted viral infection of the human central nervous system that occurs in many Eurasian countries. The TBE virus (TBEV) belongs to the genus *Flavivirus* within the family *Flaviviridae* and is phylogenetically classified into three subtypes: the Western or European subtype, the Siberian subtype, and the Far-Eastern subtype[18,20,26].

The epidemiology of TBE differs between countries according to the ecology of TBEV[3,5,20]. Forest hematophagous ticks primarily of the genus *Ixodes*, such as *Ixodes ricinus* (*I. ricinus*) and *I. persulcatus*, are vectors of TBEV and transmit the virus to competent hosts during their blood-feeding, allowing it to circulate in the environment. Wild rodents are reservoir hosts of TBEV and develop a long-lasting viremia, allowing them to infect other vector ticks and maintaining natural foci. By contrast, larger forest animals, such as cervid species and wild boars (*Sus scrofa*), and domestic animals are indicator hosts for TBEV transmission, being infested by vast numbers of vector ticks and thus facilitating transmission of the virus to new areas and circulating it through their habitats but playing no role in transmitting the virus to new ticks, probably due to them developing only brief viremia with low viral titers. Once infected, these animals produce antibodies to TBEV, which are probably maintained over a longer period[1,4,7,8,12,15].

TBE is a notifiable disease in Japan. The first human case of TBE was recorded in southern Hokkaido (the northernmost island of Japan) in 1993[22], and it was subsequently revealed that this region was a TBEV-endemic area based on the isolation of TBEV-FE strains from *I. ovatus* ticks, rodents, and dogs, as well as serological evidence in rodents, dogs, and horses [22,23,25]. Although there were no further records of TBE for a long period, three confirmed cases were reported in southern and central Hokkaido during 2016–2017[26] and a fifth patient was reported in northern Hokkaido in 2018 (http// www.city.asahikawa.hokkaido.jp/kurashi/135/ 136/150/ d064126.html; in Japanese), suggesting the widespread distribution of TBEV in Hokkaido. The location of southern, central, and northern Hokkaido areas is indicated in Fig. 1. Therefore, the examination and clarification of whether TBEV is endemic in other parts of Hokkaido such as eastern Hokkaido (Fig. 1) is urgently required to prevent the further spread of this disease. Outside Hokkaido, Shimane Prefecture in Honshu (the main island of Japan) is the only other area that has been shown to be TBEV-endemic based on a serological survey of wild rodents[25]. However, the presence of TBEV-endemic areas in other parts of Honshu has not been sufficiently examined, and few epidemiological studies have been conducted in other parts of Japan.

Indicator hosts such as cervid species and Eurasian wild boars (*Sus scrofa*) have been used as sentinels of the virus in TBEV-endemic European countries to monitor the distribution and abundance of vector ticks[2,12]. Very little is known regarding TBEV infection in these sentinel species in Japan, where sika deer (*Cervus nippon*) and wild boars are overabundant in many regions, although the latter are not present in Hokkaido[9,14,21]. However, a recent serological survey did indicate that a small number of Hokkaido sika deer (*C. n. yesoensis*) collected in northern Hokkaido during 2013 and 2014 tested positive for TBEV[24].

Raccoons (*Procyon lotor*), which are native to North America, are now widely distributed in European countries where TBEV is endemic[6,17]. However, there have been no reports of TBEV infection in raccoons in these countries. The raccoon is also an invasive species in Japan, where it has rapidly spread since the 1970s to become widely distributed throughout the country[11]. However, it is unclear whether raccoons carry TBEV-infected ticks as indicator hosts or play a role as reservoir hosts.

In this study, we investigated the prevalence

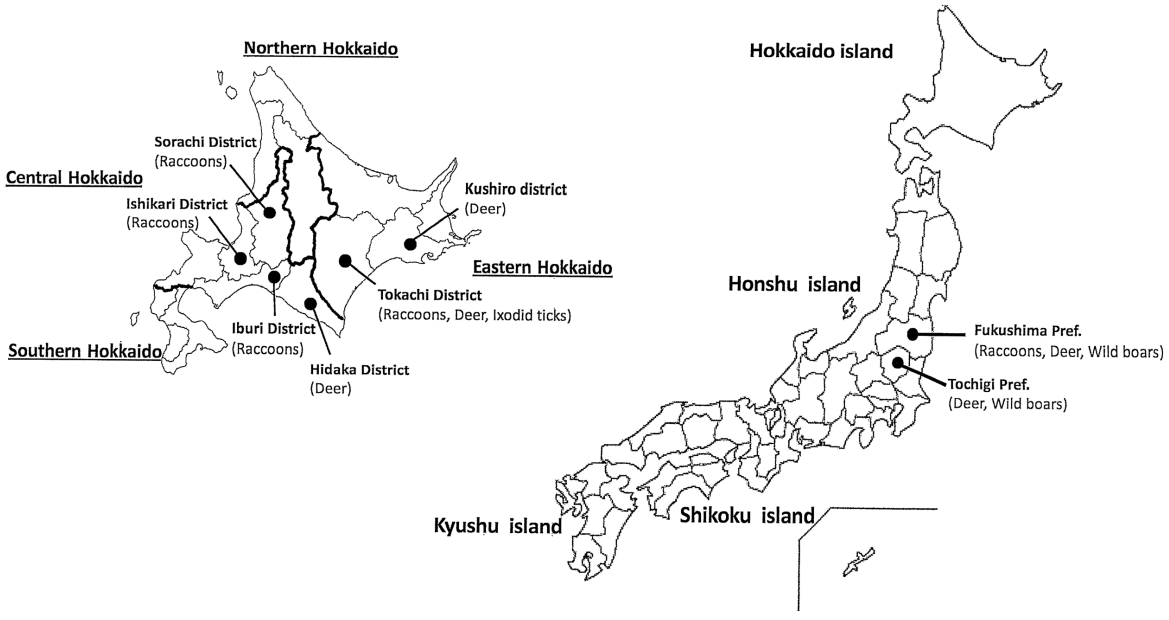

Fig. 1. Location of the sampling sites on Hokkaido and Honshu islands, Japan. The types of wild animals and ticks that were collected are indicated in parentheses. Hokkaido is divided into four areas (central, southern, northern, and eastern Hokkaido).

of TBEV in raccoons and Hokkaido sika deer in central and eastern Hokkaido using serological examinations and in ticks in eastern Hokkaido using virological examinations to define TBEV-endemic areas. In addition, we serologically examined Honshu sika deer (*C. n. centralis*), wild boars, and raccoons captured in Fukushima and Tochigi Prefectures in Honshu to identify other TBEV-endemic areas. Sampling locations, year, and numbers are described in Table 1 and Fig. 1.

Materials and Methods

Serum collection: A total of 1,649 serum samples from 1,072 raccoons in Hokkaido and Fukushima Prefectures, from 519 sika deer in Hokkaido, Fukushima, and Tochigi Prefectures, and from 58 wild boars in Fukushima and Tochigi Prefectures collected during 2003–2018 were investigated (Table 1 and Fig. 1). The detailed animal numbers captured in each sampling location (district) are provided in Table 1. Prior to testing, all of the sera were inactivated at 56°C for 30 min.

Tick collection: A total of 420 *I. ovatus* and 87 *I. persulcatus* adults were collected by flagging in forested areas in Tokachi District, eastern Hokkaido, in 2018 (Table 1 and Fig. 1). Following morphological identification of the species and sex of the ticks, they were pooled into groups of 5–10 ticks according to species, sex, and sampling location. Each pooled sample was homogenized in 1 ml of virus transfer medium containing Dulbecco's Modified Eagle's Medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% bovine serum albumin (BSA), penicillin (1,000 units/ml), streptomycin (1 mg/ml), gentamycin (100 μg/ml), and amphotericin B (20 μg/ml). The homogenates were centrifuged, and the resulting supernatants were collected and used for virus and gene detection, as described below.

Serological assays: To detect the presence of TBEV antibodies, all of the sera were first tested by enzyme-linked immunosorbent assay using recombinant TBEV subviral particles with Strep-tag (Strep-SP ELISA), as previously described[13]. Briefly, antigens were prepared by culturing human embryonic kidney 293T

Table 1. Sampling locations, years, and numbers of wild animal sera and ticks tested in this study

Species	Island	Prefecture	District	Sampling year	No. of captured animals
Raccoon	Hokkaido	Hokkaido			
		Central	Iburi	2003–2005	94
			Ishikari	2003–2005	242
			Sorachi	2003–2005	304
			Unknown[a]	2003–2005	22
		Eastern	Tokachi	2010–2018	368
	Honshu	Fukushima	Hamadori	2016–2017	35
			Nakadori	2016–2017	7
				Subtotal	1,072
Sika deer	Hokkaido	Hokkaido			
		Central	Hidaka	2010–2017	23
		Eastern	Tokachi	2010–2017	401
			Kushiro	2010–2016	13
			Unknown	2010–2017	37
	Honshu	Fukushima	Hamadori	2016	2
			Aizu	2017	1
		Tochigi	Kenhoku	2016–2018	42
				Subtotal	519
Wild boar	Honshu	Fukushima	Hamadori	2016	48
			Nakadori	2016	2
		Tochigi	Kennan	2016–2017	8
				Subtotal	58
				Total	1,649
Tick	Hokkaido	Hokkaido	Tokachi	2018	507

[a]Information was unavailable.

(HEK293T) cells transfected with the pCAG-TBE-M-StrepE plasmid expressing Strep-SPs in DMEM supplemented with 4.5 g/L D-glucose and 10% fetal bovine serum (FBS) at 37°C. The supernatant containing Strep-SPs was then concentrated by polyethylene glycol 8000 (Wako, Tokyo, Japan), as previously described[13], and the precipitated Strep-SPs were resuspended in phosphate-buffered saline (PBS) and stored at −80°C until use. Negative control antigens from the culture supernatant of non-transfected HEK293T cells were also prepared using the same procedures as described above.

Ninety-six-well ELISA plates (Thermo Fisher Scientific, Waltham, USA) were coated with 1,000-fold-diluted Precision Protein Strep-Tactin alkaline phosphatase conjugate (Bio-Rad Laboratories, Inc., Hercules, USA) in 0.1 M carbonate bicarbonate buffer (pH 9.6) and incubated overnight. After washing the plates with PBS containing 0.05% Tween-20 (PBST), they were blocked with 1% BlockAce (DS Pharma Biomedical, Osaka, Japan). The Strep-SPs and negative control antigens were then incubated on the plates at 37°C for 45 min, following which the serum samples were added (1 : 100 dilution in 1.5% BSA in PBST), and the plates were incubated for a further 45 min. Protein A/G conjugated with horseradish peroxidase (HRP) (1 : 2,000; Thermo Fisher Scientific) was then added to the raccoon and wild boar sera to detect TBEV-specific immunoglobulin G (IgG) antibodies,

whereas anti-deer IgG conjugated with HRP (1 : 500; KPL, Gaithersburg, USA) was added to the deer sera. After incubation with the conjugates for 30 min, TMB substrate (BD Biosciences, Franklin Lakes, USA) was added and the optical density (OD) was measured at 450 nm. The results were recorded as the positive/negative (P/N) ratio (i.e., the ratio of the OD value with Strep-SPs to that with the negative antigen). Serum samples with a P/N ratio of ≥1.5 were chosen for confirmation by a virus neutralization test (VNT) as described below.

VNT: VNT was performed as previously reported[13]. Briefly, serially diluted sera were mixed with an equal volume of 100 PFU/0.1 ml of the Oshima 5–10 strain of TBEV[22] at 37°C for 1 h. The mixtures were inoculated on baby hamster kidney fibroblast-21 (BHK-21) cells in 12-well plates at 37°C for 1 h, following which an overlay medium containing 1.5% carboxymethyl cellulose was added and the plates were incubated for a further 4 days. The cells were then fixed and stained with 10% formalin containing 0.1% crystal violet. Neutralizing titers (NT_{50}) of ≥1 : 50 were determined to be seropositive against TBEV.

Virus isolation: Tick homogenates were inoculated on BHK-21 cells grown in 24-well plates with 0.1 ml of sample/well and culturing them with DMEM supplemented by 2% fetal bovine serum at 37°C for 3–4 days. If no cytopathic effect was observed in the inoculated cells, the culture supernatants were passaged into newly prepared BHK-21 cells. The supernatants were also tested for TBEV RNA, as described below.

Real-time polymerase chain reaction (PCR) assay: Total RNA was extracted from the tick homogenates using Isogen LS (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, USA). The presence of TBEV RNA in ticks was detected using the real-time RT-PCR assay, as previously described[19] with slight modification. Briefly, the assay was performed on the LightCycler® Nano (Roche Diagnostics, Mannheim, Germany) using 20 µl of the EagleTaq Universal Master Mix (Roche) plus forward (F-TBE 1: 5'-GGG CGG TTC TTG TTC TCC-3') and reverse (R-TBE 1: 5'-ACA CAT CAC CTC CTT GTC AGA CT-3') primers and the TaqMan probe (TBE-Probe-WT: 5'-TGA GCC ACC ATC ACC CAG ACA CA-3') with 5 µl of the cDNA sample. Viral RNA extracted from TBEV-infected BHK-21 cells was used as a positive control. A sample was evaluated as positive if the cycle threshold value was lower than 35 cycles[19].

Statistical analysis: Fisher's exact test was used to compare the frequencies of antibodies among locations.

Results

Serological assay: Eighty-one out of 1,072 raccoon sera sampled in Hokkaido and Fukushima Prefectures were screened using Strep-SP ELISA and were found to have positive/negative (P/N) ratios of approximately ≥1.5 (Table 2). In Hokkaido Prefecture, Strep-SP ELISA-positive raccoons (78/1,030 = 7.6%) were found in all examined districts, with an incidence of 0.5%–13.6%. In Fukushima Prefecture, in contrast, Strep-SP ELISA-positive raccoons (3/42 = 7.1%) were found only in the Hamadori District. All Strep-SP ELISA-positive sera were confirmed using VNT (Table 2). As a result, 42 sera sampled in Hokkaido were VNT-positive (NT_{50} = ≥1 : 50): 39 from central Hokkaido and 3 from eastern Hokkaido. No VNT-positive sera were found among the 42 raccoons that were captured in Fukushima Prefecture.

Twenty-six out of 519 sika deer sera sampled in Hokkaido, Fukushima, and Tochigi Prefectures had P/N ratios of approximately ≥1.5 (Table 3). Moreover, 18 (4.5%) of the 474 Hokkaido sika deer captured in Tokachi District of Hokkaido

Table 2. Seroprevalence of TBEV-neutralizing antibodies in raccoons captured on Hokkaido and Honshu islands, Japan

Prefecture	District	No. of sera tested	No. of sera with[a]	
			P/N ratio of ≥1.5 (%)	VNT positivity (%)[b]
Hokkaido				
Central	Iburi	94	4 (4.2)	2 (2.1)
	Ishikari	242	10 (4.1)	5 (2.1)
	Sorachi	304	41 (13.5)	29 (9.5)
	Unknown[c]	22	3 (13.6)	3 (13.6)
	Subtotal	662	58 (8.8)	39 (5.9)
Eastern	Tokachi	368	20 (0.5)	3 (0.8)
	Total	1,030	78 (7.6)	42 (4.1)
Fukushima	Hamadori	35	3 (8.6)	0 (0.0)
	Nakadori	7	0 (0.0)	0 (0.0)
	Total	42	3 (7.1)	0 (0.0)
	Grand total	1,072	81 (7.6)	42 (3.9)

[a]A total of 81 sera with positive/negative (P/N) ratios of approximately ≥1.5 out of 1,072 sera were tested using the virus neutralization test (VNT).
[b]Data were significantly different between central Hokkaido (5.9%) and eastern Hokkaido (0.8%) ($P < 0.01$); Sorachi District (9.5%) and Iburi District (2.1%) ($P < 0.05$); and Sorachi District and Ishikari (2.1%) and Tokachi (0.8%) Districts ($P < 0.01$).
[c]Information was unavailable.

Table 3. Seroprevalence of TBEV-neutralizing antibodies in sika deer captured on Hokkaido and Honshu islands, Japan

Prefecture	District	Deer	No. of sera tested	No. of sera with[a]	
				P/N ratios of ≥1.5 (%)	VNT positivity (%)
Hokkaido Central	Hidaka	Hokkaido sika deer	23	0 (0.0)	0 (0.0)
	Unknown		37	0 (0.0)	0 (0.0)
Eastern	Tokachi		401	18 (4.5)	2 (0.5)[b]
	Kushiro		13	0 (0.0)	0 (0.0)
		Subtotal	474	18 (4.5)	2 (0.4)
Fukushima	Hamadori	Honshu sika deer	2	0 (0.0)	0 (0.0)
	Aizu		1	0 (0.0)	0 (0.0)
		Subtotal	3	0 (0.0)	0 (0.0)
Tochigi	Kenhoku	Honshu sika deer	42	8 (19.0)	1 (2.4)[c]
		Total	519	26 (5.0)	3 (0.6)

[a]A total of 26 sera with positive/negative (P/N) ratios of approximately ≥1.5 out of a total of 519 sera were tested using the virus neutralization test (VNT).
[b]These two sera had neutralizing antibody titers of 1 : 50 and 1 : 200, respectively.
[c]This serum had a neutralizing antibody titer of 1 : 50.

and 8 (19.0%) of 42 Honshu sika deer captured in Kenhoku District of Tochigi Prefecture were Strep-SP ELISA-positive; these positive sera were examined using VNT. As shown in Table 3, only 3 out of the 26 serum samples were VNT-positive: two from Tokachi District in Hokkaido and one from Tochigi Prefecture. In contrast, seropositive wild boars were not found in Fukushima and Tochigi Prefectures (data not shown).

Overall, the seroprevalence of neutralizing antibodies in raccoons was significantly higher in central Hokkaido (39/662 = 5.9%) than in

Table 4. Neutralizing antibody titers (NT_{50}) in the 42 raccoons in Hokkaido

Prefecture	District	NT_{50}		
		1 : 50	1 : 200	≥1 : 800
Hokkaido				
Central	Iburi	1	1	0
	Ishikari	3	1	1
	Sorachi	9	14	6
	Unknown[a]	1	1	1
Eastern	Tokachi	3	0	0
	Total	17	17	8

[a]Information was unavailable

eastern Hokkaido (3/368 = 0.8%) (Fisher's exact test, $P < 0.01$; Table 2). Among the districts examined, Sorachi District in central Hokkaido had significantly higher numbers of TBEV-seropositive raccoons (29/304 = 9.5%) than Iburi District (2/94 = 2.1%; $P < 0.05$) and Ishikari District (5/242 = 2.1%; $P < 0.01$) in central Hokkaido and Tokachi District in eastern Hokkaido (3/368 = 0.8%; $P < 0.01$). High antibody titers ranging from 1 : 200 to ≥1 : 800 were found in 25 (59.5%) of the 42 VNT-positive raccoons collected from central Hokkaido during 2003–2005 (Table 4). Eight raccoons had very high titers (NT_{50} ≥1 : 800), but six (75.0%) of these were captured in Sorachi District.

Sika deer had a low seroprevalence of neutralizing antibodies (3/519 = 0.6%), with only two Hokkaido sika deer sera from eastern Hokkaido being VNT-positive, with titers of 1 : 50 and 1 : 200, and one Honshu sika deer from Tochigi Prefecture in Honshu being VNT-positive, with a titer of 1 : 50 (Table 3). It was also confirmed that this TBEV-seropositive Honshu sika deer did not have neutralizing antibodies against Japanese encephalitis virus (data not shown).

Virus isolation and gene detection from ticks: Attempts were made to isolate TBEV and detect viral genes from 420 *I. ovatus* and 87 *I. persulcatus* collected in Tokachi District, eastern Hokkaido, based on the serological evidence of TBEV infection in raccoons and sika deer. However, TBEV and its viral genes were not detected in any of the tick samples.

Discussion

The first human case of TBE was reported in Japan in southern Hokkaido in 1993[22]. However, no further cases were reported until 2016–2017[26] and 2018, when four more people were diagnosed with the disease in southern, central, and northern Hokkaido (http//www.city.asahikawa.hokkaido.jp/kurashi/135/136/150/d064126.html; in Japanese). The reason for this gap is unclear, but there is now an urgent need to identify other TBEV-endemic areas to avoid future human infection with this virus. The detection of TBEV antibodies in wild and domestic animals is a very useful approach for finding potential risk areas[3,12]. Seropositive rodents, Hokkaido sika deer, dogs, and horses have been detected in southern, central, and northern Hokkaido[22–25], where human cases of the disease have also been documented, as mentioned above. Thus, the seroprevalence of TBEV in these animals is likely to become a valuable indicator of infection risk that will complement incidence-based risk assessments.

In contrast, the prevalence of the virus in ticks has proven to be an unsuitable indicator for the risk of TBEV infection because of a low virus detection rate (0%–2.7% but rarely exceeding 1%) even in large sample sizes of ticks collected from known endemic areas; additionally, ticks cannot be sampled throughout the year[12]. However, isolation of the virus would help obtain useful virologic and genetic information, resulting in the establishment of control measures for TBE.

Forest animals such as cervid species and wild boars are thought to be highly valuable sentinels for the detection of TBEV antibodies in epidemiological studies in both endemic and non-endemic areas[1,3,8,12]. However, there is a lack of serological evidence of TBEV infection in raccoons, despite their widespread distribution in

TBEV-endemic countries, including Japan[6,11,17]. In this study, we detected TBEV antibodies in raccoons collected from central and eastern Hokkaido, revealing for the first time that TBEV foci also occur in Tokachi District, eastern Hokkaido, although the seroprevalence of TBEV was significantly lower in this district (0.8%) than in central Hokkaido (5.9%) (Table 2). We also detected seropositive Hokkaido sika deer in Tokachi District, though at a low incidence rate (Table 3). The prevalence of TBEV in eastern Hokkaido has been unknown for many years. Takeda *et al.*[23] reported that the sera of horses and dogs sampled during 1992–1997 in eastern Hokkaido, including Tokachi District, tested negative for TBEV. However, no further serological surveys have been performed in this district until the present study. We also attempted to detect TBEV in ticks collected from the places where seropositive animals were found to confirm the presence of TBEV. However, the results of these tests were negative, possibly due to an insufficient sample size (n = 507)[12]. Our findings suggest that TBEV is currently distributed in Tokachi District but with a low seroprevalence. However, since the TBE case that was recorded in northern Hokkaido in 2018 occurred after TBEV antibodies were found in a small number of wild sika deer captured in 2013[24], we recommend that continuous epidemiological studies are conducted using appropriate indicator hosts to monitor existing and detect new TBEV foci.

Several serological surveys have been conducted in horses, dogs, and deer in central Hokkaido[23,24]. However, TBEV antibodies were only detected in a small number of dog serum samples collected from one of the five districts examined (Iburi) in 1994–1997[23]. By contrast, in the present study, we detected antibodies in raccoons captured in all of the districts tested (Iburi, Ishikari, and Sorachi) during 2003–2005 (Table 2), as well as in eastern Hokkaido (Tables 2 and 3). Furthermore, the recent TBE cases were reported in central Hokkaido in 2017[26] and in northern Hokkaido in 2018 (http//www.city.asahikawa.hokkaido.jp/kurashi/135/136/150/d064126.html; in Japanese). Therefore, it appears that TBEV-endemic areas may have been widely distributed throughout Hokkaido for a long time.

Igota *et al.*[10] reported that Hokkaido sika deer had summer home ranges that were widely scattered over 5,734 km^2 and migration distances of up to 100 km. Therefore, the detection of seropositive deer in a particular area may not necessarily imply the presence of TBEV foci, as it is possible that these deer migrated from outside the area[24]. By contrast, raccoons have a much smaller home range that varies from 0.3 km^2 in urban areas to 22 km^2 in forests[11]. Therefore, we believe that raccoons are a particularly valuable sentinels of TBEV that will be useful for discovering unknown TBEV-endemic areas.

We cannot rule out the possibility that raccoons will become a reservoir host for the maintenance and amplification of TBEV in natural foci, however. High titers of TBEV antibodies were observed in bank voles (*Myodes glareolus*) with high TBEV RNA loads for a long period[16] and, in the present study, we found that several raccoons had high antibody titers of $\geq$ 1 : 800 (Table 4). Therefore, although the relationship between the viremia level and antibody level is not clear, these infected raccoons may have developed a high and persistent viremia level. However, experimental infection with raccoons should be considered to test this hypothesis.

In an epizootiological study of TBEV infection in rodents on Honshu in 1997–2003, seropositive rodents were only detected in Shimane Prefecture (Honshu)[26]. In this study, we detected seropositive Honshu sika deer in Tochigi Prefecture (Honshu) (Table 3), indicating the presence of TBEV foci in this area. Although there have been no confirmed cases of TBE in any part of Japan except Hokkaido, these results indicate that further investigations are required to better understand the distribution of TBEV in this country.

In conclusion, we found that both raccoons and sika deer may be valuable indicator hosts of

TBE that will help us to discover natural foci of the disease, as both species allowed us to identify new TBEV-endemic areas in Hokkaido and Honshu. A relatively high seroprevalence of TBEV was found in raccoons collected from areas of Hokkaido where the TBE cases to date have been reported. Since the number and distribution of raccoons have increased in Hokkaido, and raccoons can gain access to residential areas with relative ease[11] (http://www.pref.hokkaido.lg.jp/ks/skn/araiguma/genjyo28Ver2.pdf, in Japanese), the connection between raccoon populations and TBE occurrence should be assessed. Together, our findings highlight the value of serological surveys using wild animals as sentinels for revealing TBEV natural foci and avoiding emerging TBE.

Acknowledgements

We would like to thank municipalities for collecting raccoon samples and the Emerging and Re-emerging Infectious Diseases Unit members in Obihiro University of Agriculture and Veterinary Medicine for their assistance. We would also like to thank Ms. Sachiko Matsuda of our unit for her excellent technical assistance. This work was partly supported by JSPS KAKENHI Grant Number JP15K07737 of the Japan Society for the Promotion of Science (JSPS). This work was also partly supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan as a project for Joint Usage/Research Center and AMED under Grant Number JP18fk0108036h0002.

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